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Immunomodulatory effects of the ether phospholipid edelfosine in experimental autoimmune encephalomyelitis

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Abstract

The 2-lysophosphatidylcholine analog edelfosine induces apoptosis in highly proliferating cells, e.g. activated immune cells. We examined mechanisms of action of edelfosine on immune functions in experimental autoimmune encephalomyelitis, a well-accepted animal model for multiple sclerosis. We observed activated caspase-3 expression in lymphoid organs and the central nervous system, however, edelfosine did not induce global apoptosis. Edelfosine improved the disease course and led to reduced frequencies of $CD4^+$ T cells infiltrating into the central nervous system. Our data suggest edelfosine as an interesting treatment candidate for multiple sclerosis.

Keywords

edelfosine, experimental autoimmune encephalomyelitis, multiple sclerosis, T cells, apoptosis

1. Introduction

Apoptotic cell death is an elementary cellular response and involves the sequential activation of caspases. The stress-inducible, intrinsic apoptotic pathway involves cytochrome c release from mitochondria (Liu et al., 1996). The extrinsic pathway is induced by pro-apoptotic and pro-inflammatory cytokines, e.g. FasL, TRAIL and TNF-α, respectively, which induce the intracellular formation of specific death-inducing signaling complexes (DISCs) after binding to death domain receptors (Micheau and Tschopp, 2003; Scaffidi et al., 1999). Downstream caspases, e.g. caspase-3, are cleaved to execute cell death. Importantly, elimination of cells via apoptosis does not lead to inflammation or immune activation. Therefore, approaches to induce apoptosis in deregulated, e.g. self-reactive immune cells have been considered to treat autoimmune diseases, for instance multiple sclerosis (MS).

Many potentially pathogenic factors and disease mechanisms have been examined in animal models, particularly in experimental autoimmune encephalomyelitis (EAE) (Raine et al., 1980), and both animal and human studies point to a central role for autoreactive $CD4^+$ T cells in MS pathology (Sospedra and Martin, 2005). $CD4^+$ T cells are present in central nervous system (CNS) and cerebrospinal fluid (CSF) cellular infiltrates in both MS and EAE (Martin et al., 1990; Ota et al., 1990; Pettinelli and McFarlin, 1981; Richert et al., 1983). Further, albeit indirect evidence for their role stems from the observation that a large fraction of the genetic risk of MS is conferred by HLA-class II alleles of the HLA-DR15 haplotype (Hillert and Olerup, 1993; Jersild et al., 1973). Recently published large genome-wide association studies show over 130 single nucleotide polymorphisms besides HLA-DR as risk alleles for MS (Beecham et al., 2013; Sawcer et al., 2011), and interestingly many of these are involved in T cell activation and function (Lundmark et al., 2007; McElroy and Oksenberg, 2008).

Following priming with (auto)antigens to potentially autoreactive $CD4^+$ T cells in the periphery, activated $CD4^+$ T cells transmigrate across the blood brain barrier (BBB) and the

blood CSF barrier (Cannella and Raine, 1995). Next, T cells are locally reactivated by antigen presenting cells within the CNS (Flügel et al., 2001). Subsequently, a number of immune cells including those of the innate (neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells) and adaptive ($CD4^+$ and $CD8^+$ T cells, B cells) immune system (Franciotta et al., 2008; Hauser et al., 2008; Lucchinetti et al., 1996) are recruited and form focal inflammatory lesions (Simpson et al., 1998). Immunodominant peptides of myelin proteins, e.g. myelin oligodendrocyte glycoprotein (MOG) peptide $MOG_{(35-55)}$, are used to induce chronic EAE in C57BL/6 mice, whereas relapsing-remitting EAE (RR-EAE) in SJL mice follows the injection of proteolipid protein (PLP) peptide $PLP_{(139-154)}$.

The synthetic 2-lysophosphatidylcholine (LPC) analog edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ET-18-OCH_3) was found to induce selective apoptosis in leukemic cells (Diomede et al., 1993; Mollinedo et al., 1993). In contrast to other cytotoxic drugs edelfosine does not target the DNA and it does not directly interfere with the formation and function of the cellular replication machinery. Edelfosine binds to Fas/CD95 in a lipid raft-mediated process thereby exerting its cytotoxic activity (Gajate and Mollinedo, 2001; van der Luit et al., 2002). Detailed mechanistic studies have demonstrated that edelfosine-treated Jurkat leukemic T cells undergo apoptosis following recruitment of DISCs into lipid rafts (Gajate et al., 2009). Edelfosine accumulates in the inner leaflet of the plasma membrane in lipid rafts, which in turn induces the clustering of rafts and the recruitment of Fas into rafts with subsequent translocation of Fas, FADD and caspase-8 into rafts, DISC formation and activation of caspase-8 to induce apoptosis in the absence of FasL. Normal, resting cells are not able to take up significant amounts of edelfosine (Gajate et al., 2000). Additionally, edelfosine may accumulate in lipid rafts within the plasma membrane followed by endocytosis and translocation to the intracellular location of the CTP:phosphocholine cytidylyltransferase (CCT), the endoplasmic reticulum (ER) (Clement and Kent, 1999; van der Luit et al., 2007, 2002). Here, edelfosine may inhibit the biosynthesis of

phosphatidylcholine (PC) leading to mitotic arrest and apoptosis (Boggs et al., 1995; Van Der Luit et al., 2003). This effect was found in exponentially growing cells, which require high amounts of PC, while normal quiescent cells were not affected (van der Sanden et al., 2004; Zerp et al., 2008). The relative contribution of each mechanism may depend on the cell type and the concentration of edelfosine (Mollinedo et al., 1997; Tsutsumi et al., 1998; Zoeller et al., 1995). Thus, alkyl lysophospholipids like edelfosine may affect several cellular processes, probably with cell type-dependent emphasis but with the joint outcome of apoptosis induction.

Due to its immunomodulatory properties and its oral availability edelfosine had already been examined as a treatment for MS in the past (Klein-Franke and Munder, 1992; Munder and Westphal, 1990). Although not performed according to today's standards exploratory clinical trials demonstrated the drug's excellent safety profile. Parallel EAE studies, mostly with rats, but also with mice described a beneficial effect of edelfosine treatment on clinical outcome (Baker et al., 1991; Chabannes et al., 1992; Klein-Franke and Munder, 1992; Kovarik et al., 1995). So far, however, functional investigations on edelfosine-induced modifications of cellular responses in the context of EAE are limited. We therefore wanted to reassess and expand prior work in the EAE model to examine in more detail the mechanism/s of action of edelfosine as a potential treatment in autoimmune diseases, for instance MS.

2. Materials and Methods

2.1 Ethics statement

All animal experiments were performed in accordance with the guidelines of the local authorities (Behörde für Soziales, Gesundheit und Verbraucherschutz Hamburg; G22/08).

2.2 Preparation of edefosine

For *in vivo* applications, edelfosine (Medmark, Oberhaching, Germany) was dissolved in aqua ad injectabilia and diluted with PBS (PAA, Pasching, Austria). The diluted edelfosine was stored at -20°C and thawed on the day of application. The injected edelfosine dose per mouse was 1 mg/kg, 10 mg/kg, 15 mg/kg and 25 mg/kg edelfosine. Edelfosine was applied by intraperitoneal injection or by gavage.

2.3 Mice

C57BL/6J mice were purchased from the Jackson Laboratory and bred by the animal facility of the University Medical Center Hamburg Eppendorf. SJL/JHan^{TM}Hsd mice were purchased from Harlan Laboratories, Indianapolis, IN, USA.

2.4 Induction of EAE

For EAE experiments C57BL/6 mice or SJL mice were housed in "individually ventilated cages (IVC)"-racks at least one week prior to the active induction of EAE. On the day of immunization mice were injected subcutaneously at two sites of the flanks with 200 µg of $MOG_{(35-55)}$ (NeoMPS, San Diego, CA, USA) or 75 µg/ml of $PLP_{(139-151)}$ (NeoMPS) peptide in incomplete Freund's adjuvant (BD Difco Diagnostics, Sparks, MD, USA) supplemented with 4 mg/ml Mycobacterium tuberculosis H37 Ra (BD Difco). C57BL/6 mice were injected intravenously with 300 ng of pertussis toxin (Calbiochem Merck, Darmstadt, Germany) on the day of immunization and 48 h later. SJL mice received a single dose of 200 ng pertussis toxin on the day of immunization. Body weight and clinical score were monitored on a 0 to 5 scale with classifications of disease severity: 0 = healthy, 1 = limp tail, 2 = ataxia and/or paresis of hind limbs, 3 = paraplegia, 4 = paraplegia with forelimb weakness, 5 = moribund or dead.

2.5 Preparation of cells from secondary lymphoid organs and CNS of mice

For preparation of lymph nodes and spleens mice were sacrificed at day 9 after immunization. Single cell suspensions were prepared by mincing the tissue and pushing it through a 40 µm cell sieve. Cells derived from the spleen were resuspended in 5 ml red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in ddH_2O) and incubated on ice for 7 min. Cells were resuspended in FACS buffer (0.1% BSA, 0.02% NaN_3 in PBS) or PBS + 1% FCS if the preparation was followed by recall experiments. At the acute phase of EAE mice were perfused transcardially with 50 ml PBS supplemented with 1% FCS to prepare the CNS. Dissected brain and spinal cord were digested for 60 min at 37°C by addition of 1 mg/ml collagenase I and 0.1 mg/ml DNase I (both from Roche, Penzberg, Germany) in D-MEM (Gibco, Carlsbad, CA, USA). The cell suspension was passed through a 40 µm cell strainer and centrifuged (250xg, 10 min, 4°C). Cells were separated from myelin and neurons by Percoll (GE Healthcare, Chalfont St. Giles, UK)-gradient centrifugation (30%/78%). CNS-infiltrating cells and microglia were collected from the interface. Cells were resuspended in FACS buffer, centrifuged (550xg, 10 min, 4°C) and washed two additional times (250xg, 10 min, 4°C).

2.6 Cell culture experiments

To determine T cell proliferation by [methyl-^{3}H]-thymidine incorporation, spleen- and lymph node-derived cells were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) at $2x10^5$ cells/well. Murine cells were cultured in 200 µl of complete mouse medium (50 µM β-mercaptoethanol, 10% FCS in RPMI1640 medium, GlutaMAX™ (Gibco)) supplemented with the disease-relevant peptide $PLP_{(139-151)}$, a polyclonal anti-CD3 antibody (145-2C11, from eBioscience, San Diego, CA, USA) or mitogenic Concanavalin A (Con A, from Sigma-Aldrich, St. Louis, MO, USA).

2.7 Immunohistochemistry

Mice were transcardially perfused with cold PBS followed by 4% cold PFA (AppliChem, Darmstadt, Germany) in phosphate buffer. Preparation of brain, spinal cord and optic nerves was followed by post-fixation of the tissue in 4% PFA in phosphate buffer (30 min, 4°C). The tissue was impregnated 2-3 days in 30% sucrose in PBS for cryoprotection. Cervical, thoracic and lumbar spinal cord as well as cerebellum and forebrain were separated. The tissue was embedded in Tissue Freezing Medium (Jung, Leica Biosystems, Nussloch, Germany) and frozen in isopentane (Carl Roth, Karlsruhe, Germany). Tissue was stored at -80°C. 12-14 µm slices were generated using a cryostat and mounted on glass microslides (Superfrost/Plus from Karl Hecht, Sondheim, Germany). For quantification of Neuronal Nuclei (NeuN)-expressing neuronal cells within the cervical spinal cord 14 µm cryosections were rinsed 3x with PBS and incubated for 2 h at RT in blocking solution (0.1% Triton X 100 (Carl Roth), 10% normal donkey serum (Chemicon, Billerica, MA, USA) in PBS). Subsequently, primary anti-NeuN antibody (A60, Millipore, Billerica, MA, USA) was added, and the sections were incubated at 4°C over night. The tissue was rinsed 3x with PBS and incubated with DyLight488-labeled secondary antibody (Jackson ImmunoResearch, Newmarket, UK). Anti-NeuN antibody was diluted 1:100 in PBS, the secondary antibody was diluted 1:400 in PBS. For each mouse, six cervical spinal cord sections were selected and two adjacent photographs per ventral horn were made. Quantification was carried out by detection of $NeuN^+$ neurons within the ventral horn including the lower motor neurons. Cervical spinal cord sections from age-matched, not-immunized SJL control mice were used to rank EAE-induced neuronal loss and edelfosine-mediated treatment effects. Images were acquired using a Axiovert 40 inverted microscope (Carl Zeiss, Jena, Germany) and analyzed with AxioVision AxioVs40 V 4.6.3.0 software (Zeiss). Photographs were taken with 20x magnification. $NeuN^+$ cells were counted and the average number of neurons per section was calculated.

2.8 [methyl-^{3}H]-thymidine-incorporation assay

T cells were derived from lymph nodes and spleens of SJL mice that were immunized as previously described. T cell proliferation was determined by the incorporation of [methyl-^{3}H]-thymidine (Hartmann Analytic, Braunschweig, Germany) after 72 h of incubation. 16 h before harvesting the cells, 1 µCi [methyl-^{3}H]-thymidine was added to each well. For quantification of beta particle emission the cells were harvested, washed and analyzed by using a beta counter (Wallac, Waltham, MA, USA). The stimulation index (SI) is calculated by dividing the mean counts per minute (cpm) of stimulated cells by cpm of respective unstimulated controls.

2.9 Flow cytometry

For the analysis of intracellular cytokines cells were incubated for 4 h in complete mouse medium supplemented with 100 ng/ml PMA and 1 µg/ml ionomycin (both from Sigma-Aldrich). After 1 h 10 µg/ml Brefeldin A (BioLegend, San Diego, CA, USA) was added. For Fc-receptor blocking cells were incubated in FACS buffer supplemented with anti-CD16/CD32 antibody (93, eBioscience). CNS-infiltrating leukocytes were quantified using BD Trucount Absolute Counting Tubes (BD Biosciences, San Jose, CA, USA) and anti-CD45 antibody. For intracellular staining of cytokines, but also of activated caspase-3, IC fixation buffer and permeabilization buffer (eBioscience) were used. Staining for Foxp3 was performed according to the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). For exclusion of dead cells from the preparation of CNS-infiltrating immune cells, the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) was used. The following antibodies were used to perform analysis by flow cytometry: anti-CD3-PacificBlue (500A2), anti-NK1.1-PE-Cy7 (PK136) and anti-caspase-3-PE (C92-605) from BD Pharmingen (San Diego, CA, USA), anti-CD3-PerCP-Cy5.5 (145-2C11), anti-CD4-FITC (GK1.5), anti-CD8a-PE-Cy7 (53-6.7), anti-CD45-APC-Cy7 (30-F11) from BioLegend, as

well as anti-CD4-APC-eFluor780 (RM4-5), anti-CD4-eFluor450, anti-CD4-PE (GK1.5), anti-CD8a-eFluor450 (53-6.7), anti-CD11b-APC (M1/70), anti-CD11b-FITC (M1/70), anti-CD11c-APC (N418), anti-CD11c-PE-Cy7 (N418), anti-CD25-APC (PC61.5), anti-CD44-APC (IM7), anti-CD45-PE-Cy7 (30-F11), anti-CD45R(B220)-PE-Cy5.5 (RA3-6B2) anti-CD62L-PE-Cy7 (MEL-14), anti-CD69-FITC (H1.2F3), anti-IFN-γ-PE (XMG1.2), anti-IL-17A-Alexa647 (17B7), anti-Ly-6G-FITC (RB6-8C5), anti-Ly-6G-PE (RB6-8C5) and anti-NK1.1-PE (PK136), all from eBioscience. Data was acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with BD FACSDiva and FlowJo (Tree Star, Ashland, OR, USA) software.

2.10 Statistical analysis

EAE disease courses were analyzed by two-way ANOVA and Bonferroni multiple testing correction (post-hoc analysis) using Prism 5.02 software (GraphPad Software, La Jolla, CA, USA). In graphs representing the EAE disease courses treatment effects were indicated by asterisks. Significant differences between groups were described in respective figure legends after Bonferroni post-hoc analysis. Cumulative disease scores and neuronal quantification by immunohistochemistry were analyzed by 1-way ANOVA followed by Bonferroni post-hoc test with asterisks indicative of significant differences between groups. Moreover, data generated by flow cytometry for leukocyte subsets derived from EAE-induced mice were evaluated by Bonferroni post-hoc analysis after 1-way ANOVA. With regard to lymph nodes and spleen-derived subsets, frequencies were normalized and expressed as fold-changes in order to allow merging of two independent experiments. *Ex vivo* proliferation assays of murine lymph node cells were analyzed by 1-way ANOVA and Bonferroni post-hoc analysis, whereas results of proliferation assays of spleen-derived cells were tested by non-parametric Kruskal-Wallis test followed by Dunns post-hoc analysis.

3. Results

3.1 Edelfosine treatment improves clinical symptoms in the EAE mouse model

In a first dose finding study we examined edelfosine in the chronic, MOG peptide-induced EAE model in C57BL/6 mice (Fig. 1A). From the day of immunization (day 0) mice were administered with equal volumes of edelfosine or PBS by gavage on a daily basis. The PBS-treated control cohort developed first signs of EAE at day 12 after immunization reaching maximum EAE score at day 18. This acute phase of disease was shifted to later days if mice were treated with edelfosine. In contrast to higher doses the treatment of mice with 10 mg/kg edelfosine did not lead to apparent side effects, e.g. tremor, inactivity, and slowed motion, but resulted in a reduced EAE score. Administration of edelfosine led to a significant amelioration of EAE when comparing PBS-treated controls and mice treated with 10 mg/kg edelfosine. We therefore continued with 10 mg/kg as effective edelfosine concentration.

In a subsequent preventive approach we studied the effects of edelfosine on RR-EAE disease course in SJL mice. SJL mice were immunized with the $PLP_{(139-151)}$ peptide (day 0). Notably, the treatment of mice with edelfosine from day 5 every other day by i.p. injection did not result in significant differences between cohorts regarding treatment effects in the course of EAE (Fig. 1B) and regarding cumulative disease scores (Fig. 1D) despite a trend to improved disease scores. To investigate the therapeutic effectiveness of edelfosine on EAE, SJL mice were immunized and disease scores were monitored. Mice showed first distinct and evenly distributed signs of EAE across groups at day 11. Treatment with PBS, 1 mg/kg edelfosine, or 10 mg/kg edelfosine was started at this day and continued until day 45 (Fig. 1C). Clinical scores upon edelfosine treatment were reduced, however, the treatment effect did not reach statistical significance despite a clear tendency towards reduced cumulative disease scores in

edelfosine-treated cohorts (a significant treatment effect with significant differences between PBS-treated and 10 mg/kg edelfosine-treated groups was detectable for the therapeutic treatment setting if EAE scores were analyzed until day 20), summarized in Fig. 1E.

However, the preventive edelfosine treatment of SJL mice was effective when started at day 0. Immediately after EAE induction PBS, 1 mg/kg edelfosine, or 10 mg/kg edelfosine was administered and repeated on a daily basis by i.p. injection. The progression of disease course was monitored until day 40 (Fig. 2A). A significant treatment effect was found in this preventive setting. Interestingly, differences emerged at the first disease bout from day 12 to 14 as well as at the first EAE relapse from day 28 to 33. A significant treatment effect was also detected when the cumulative disease scores of the three cohorts were analyzed (Fig. 2B). Differences in post-hoc analysis were found for the comparison of PBS-treated controls to 10 mg/kg edelfosine-treated mice.

3.2 Preventive edelfosine treatment increases activated caspase-3 expression in the preclinical phase of RR-EAE

For a detailed analysis of the impact of edelfosine on different immunological parameters immunized SJL mice received PBS, 1 mg/kg edelfosine, or 10 mg/kg edelfosine by i.p. injection from the day of immunization (day 0) on a daily basis until day 9. There were no differences in absolute cell numbers in spleens as well as draining lymph nodes during the preventive treatment (data not shown). Furthermore, neither immune cell subset derived from spleens nor subsets derived from lymph nodes displayed any edelfosine treatment-associated changes in frequencies compared to PBS-treated controls ($CD3^+$ T cells, $B220^+$ B cells, $CD11b^+CD11c^-$ macrophages, $Ly\text{-}6G^+CD11b^+$ neutrophils, $CD11b^-CD11c^+B220^-$ myeloid DCs, $NK1.1^+CD45^+CD11b^+CD3^-$ NK cells, $CD11b^-CD11c^{low}B220^+Ly\text{-}6G^{low}$ plasmacytoid DCs, data not shown). The gating strategy is shown in Supplementary Fig. 1. Further, no

differences in global $CD4^+$ and $CD8^+$ T cells were observed (Fig. 3A and -B), while naïve $CD4^+CD62L^{high}CD44^{low}$ T cells increased in lymph nodes upon edelfosine treatment (Fig. 3C). No differences were seen between frequencies of $CD4^+CD69^+$ or $CD4^+CD25^+$ T cells isolated from spleens and lymph nodes in either group or for the respective $CD8^+$ T cell populations (data not shown). Thus, edelfosine treatment did not influence frequencies of T cells in the periphery, which express strong activation signals. As an additional $CD4^+$ T cell subset $CD4^+CD25^+Foxp3^+$ natural regulatory T cells (nTregs) were identified in spleens and lymph nodes of EAE-induced mice by gating on $CD4^+CD25^+$ populations excluding $CD69^+$ events. The comparison of nTreg frequencies from PBS-treated and edelfosine-treated cohorts in either spleens or lymph nodes did not indicate treatment-dependent changes (data not shown). The treatment of EAE-induced mice with edelfosine was not found to have an impact on IFN-γ or IL-17-producing $CD4^+$ as well as $CD8^+$ lymphocyte subset frequencies in the spleen or lymph nodes compared to controls (data not shown). Hence, it does not appear that edelfosine leads to global perturbations of the peripheral immune system.

Since it had previously been demonstrated that elimination of T cells from the brain in the EAE model involves apoptosis (Schmied et al., 1993), and since this is the presumed mechanism of action of edelfosine, we wanted to examine this aspect by caspase-3 activation as an early step of apoptosis upon edelfosine treatment. Therefore, $CD4^+$ and $CD8^+$ T cells prepared from spleens and lymph nodes of EAE-induced mice that were treated with PBS or edelfosine were stained for caspase-3 activation (Fig. 4A and -B). As edelfosine is described as acting primarily on proliferating cells, CD69 was used to specifically determine activated T cells. In spleens the treatment of EAE-induced mice with 10 mg/kg edelfosine led to a significant increase in frequencies of $CD4^+$ (1.70-fold) and $CD8^+$ (1.99-fold) T cells with activated caspase-3 compared to PBS-treated, immunized mice (Fig. 4C). 10 mg/kg edelfosine also resulted in a 1.59-fold increase in frequencies of $CD4^+$ T cells with activated caspase-3 compared to 1 mg/kg edelfosine. Additionally, activated caspase-3-expressing

$CD69^+CD8^+$ T cell frequencies were elevated in spleens after 10 mg/kg edelfosine treatment (2.04-fold compared to PBS, 1.95-fold compared to 1 mg/kg edelfosine).

3.3 Edelfosine treatment does not compromise the proliferative capacity of T cells in RR-EAE

Spleen and lymph node cells of edelfosine-treated mice retained their proliferative capacity as shown in *ex vivo* proliferation experiments. Of interest, *ex vivo* activation with $PLP_{(139-151)}$ appears to indicate an edelfosine dose-dependent effect on proliferation *in vivo* (Fig. 5A). Increased SIs were found at higher doses of edelfosine (Fig. 5B). This phenomenon is caused by the greater reduction of background proliferation (in the absence of antigenic stimulus *in vitro*) as a result of edelfosine treatment of mice (Fig. 5A and -B).

3.4 Edelfosine treatment reduces CNS-infiltrating T cell frequencies and leads to expression of activated caspase-3

The acute phase of EAE is clinically apparent through maximal impairment of motor function. Pathologically, this period is characterized by a marked infiltration of immune cells from the periphery into the CNS. In this set of experiments SJL mice were immunized with $PLP_{(139-151)}$ to induce EAE. Mice were either treated with PBS, 1 mg/kg edelfosine, or 10 mg/kg edelfosine from the day of immunization on a daily basis. PBS-treated mice displayed a maximum mean EAE score of 2.67 ± 0.51 at day 14 after immunization (Supplementary Fig. 2A). By contrast, mice treated with 1 mg/kg or 10 mg/kg edelfosine showed a considerably lower clinical score (1.63 ± 0.42 and 0.13 ± 0.13, respectively). At day 15 spinal cords and brains were prepared to analyze cellular infiltration of the CNS by flow cytometry (Supplementary Fig. 2B and 2C, Supplementary Fig. 3). No significant differences of absolute

cell numbers in the CNS were detected between PBS-treated mice and at both doses of edelfosine (Supplementary Fig. 2B). 10 mg/kg edelfosine resulted in reduced frequencies (-1.71-fold) of $CD3^+$ T cells in the CNS (Supplementary Fig. 2D) and at the same time increased frequencies (6.46-fold) of neutrophils compared to PBS controls. $CD4^+$ T cell frequencies were reduced at 10 mg/kg edelfosine in comparison to 1 mg/kg edelfosine or control (-1.17-fold) (Fig. 6). In contrast, no differences were determined for $CD8^+$ T cells (Fig. 6). Compared to PBS edelfosine did not alter the frequency of CNS-infiltrating nTregs (Fig. 6) or IFN-γ-producing $CD4^+$ T cells, while IFN-γ-producing $CD8^+$ T cells were reduced (Fig. 7). Edelfosine led to a significant treatment effect in view of IL-17-producing $CD4^+$ T cells, but no differences could be detected between groups after post-hoc analysis (Fig. 7). Finally, the impact of edelfosine treatment on apoptosis induction, i.e. caspase-3 activation, in CNS-infiltrating T lymphocyte subsets was investigated (Fig. 8), and a clear increase of caspase-3-expressing $CD4^+$ T cells (4.63-fold) was detected at 10 mg/kg edelfosine compared to PBS-treated controls. No differences were detected for caspase-3 activation in $CD8^+$ T cells.

3.5 Preventive edelfosine treatment reduces neuronal loss in acute RR-EAE

To detect and quantify the damage of neurons within the cervical spinal cord of EAE-affected SJL mice during the acute disease phase, tissue sections were prepared for immunohistochemistry. Sections were stained with antibodies against the neuron-specific nuclear protein NeuN (Fig. 9). Compared to controls a significant decrease in $NeuN^+$ neurons was detected in EAE-induced, PBS-treated mice. Neuronal loss could be reduced markedly by treatment with 1 mg/kg or 10 mg/kg edelfosine.

4. Discussion

The main findings of our study are the clinical amelioration of RR-EAE by systemic edelfosine treatment and, at the level of its mechanism, the increase of apoptosis-prone, activated caspase-3-expressing $CD4^+$ T cells within the CNS of EAE-induced mice. Additionally, proliferative T cell responses upon recall stimulations were not impaired, and neuronal damage was decreased upon treatment. Our results, therefore, translate *in vitro* generated mechanistic knowledge about edelfosine into the *in vivo* settings.

10 mg/kg edelfosine was identified to be effective in ameliorating chronic-progressive EAE without causing side effects after preventive, oral administration. Additionally, EAE incidences were reduced upon edelfosine treatment confirming observations in Biozzi AB/H mice (Baker et al., 1991). In accordance with these previous EAE trials in Biozzi AB/H mice, 10 mg/kg edelfosine treatment resulted in delayed onset of EAE. The beneficial effect of 10 mg/kg edelfosine was subsequently validated in the relapsing-remitting EAE model in SJL mice. As experiments by Klein-Franke and Munder pointed to the effectiveness of treatment initiation from day 5 after immunization, SJL mice received edelfosine from day 5 in a second EAE experiment (Klein-Franke and Munder, 1992). Additionally, dose rates were limited by edelfosine treatment only every other day. Interestingly, pharmacokinetic studies in rats reported a rapid uptake and distribution of a liposomal formulation of an edelfosine L-isomer into tissues (time to reach highest concentrations: 0.25 to 8 h, half-lives were 13.1 h in blood and 14 h in spleens) (Bhamra et al., 2003). Furthermore, approximately 96% of edelfosine was absorbed in the first 24 hours after oral treatment of rats (Kötting et al., 1992). These and our data denote that edelfosine given every 48 h may not be suitable for maintaining a local concentration necessary to interfere with the priming of autoreactive immune cells in EAE and that intervals need to be reduced to balance clearance of edelfosine from peripheral lymphoid organs. In the preventive setting, daily treatment was adequate, probably by

sustaining edelfosine concentrations in peripheral lymphoid organs, which were effective to interfere with immune cell functions. These results suggest that edelfosine acts not only via modulation of T cell priming for EAE induction but also on induction of relapses.

LPC and its synthetic analogs were initially described to enhance the phagocytic activity of macrophages (Burdzy et al., 1964; Munder et al., 1969, 1966). Therefore they were assigned an immunomodulatory role in defense mechanisms of the immune system. Since MS but also EAE are mediated by myelin-specific encephalitogenic T cells (Coles et al., 1999; Martin et al., 1992; Wekerle et al., 1986; Zamvil and Steinman, 1990), treatment effects on T cell priming in secondary lymphoid tissues were determined. Edelfosine does not act by indiscriminately eliminating cells in secondary lymphoid organs that are indispensable for priming and triggering antigen-specific immunity, i.e. edelfosine does not lead to major perturbations or loss of peripheral immune cells. Importantly, edelfosine treatment did not induce the ablation of nTregs from peripheral lymphoid organs. Tregs are thought to be involved in preventing the development of autoimmune diseases, and changes in number and function were also reported in MS (Haas et al., 2005; Viglietta et al., 2004) and EAE (McGeachy et al., 2005; Yu et al., 2005; Zhang, 2004). The frequencies of nTregs after repeated edelfosine treatment were not altered indicating that it does not affect nTreg-mediated peripheral tolerance mechanisms. To confirm the previously shown apoptosis induction by edelfosine the frequencies of caspase-3-activated $CD4^+$ and $CD8^+$ T cells were evaluated. The *ex vivo* analysis of peripheral T cells from edelfosine-treated, EAE-induced mice demonstrated increased frequencies of apoptosis-prone T cells.

To further delineate the influence of daily edelfosine treatment on the functional properties of spleen and lymph node-derived T cells, cells were used in *ex vivo* restimulation experiments. Repetitive edelfosine treatment of mice was not found to interfere with the capacity of lymphocytes to proliferate and to respond to inflammatory cues independent of the stimulus (mitogenic, polyclonal or antigen-specific). Upon each of the stimuli no significant change in

proliferation was observed by daily edelfosine treatment confirming that edelfosine does not compromise the proliferative potential of T cells. These findings are of practical and translational relevance, since some of the approved immunomodulatory drugs for MS, e.g. mitoxantrone and alemtuzumab to name two approved drugs, eradicate a wide spectrum of immune cells or broadly inhibit immune function. Such therapies carry the risk of various side effects, among which the increase in susceptibility to infections.

In EAE, impairment of motor function is maximal during acute disease, and edelfosine ameliorated the functional deficits as shown by reduced EAE scores. One curious observation is the increase of neutrophils in the CNS of mice receiving 10 mg/kg edelfosine. While we can only speculate on the causes of this finding, edelfosine crosses the BBB (Arnold et al., 1978; Bhamra et al., 2003; Estella-Hermoso de Mendoza et al., 2009). Drug concentrations that are achieved by administration of 10 mg/kg edelfosine treatment may be sufficient to induce activation in neutrophils. Neutrophils can be activated *in vitro* by platelet activating factor (PAF) (Read et al., 1993), and interestingly, PAF is a natural analogue of edelfosine. PAF has been involved in inflammatory processes like neutrophil chemotaxis (Chignard et al., 1979; O'Flaherty et al., 1981). Human neutrophils showed increased cytosolic free Ca^{2+} concentration mediated by edelfosine binding to PAF-R, but the affinity was 5000-fold lower compared to PAF (Alonso et al., 1997). Mature peripheral blood neutrophils were not susceptible to edelfosine-induced apoptosis (Mollinedo et al., 1997). Thus, edelfosine may not be able to induce apoptosis but rather to activate neutrophils due to molecular similarities with its physiological counterpart PAF. EAE is mainly mediated by $CD4^+$ T cells, and the specificity of autoreactive T cells for distinct myelin peptides has been studied in detail (McRae and Miller, 1994; McRae et al., 1995; Pettinelli and McFarlin, 1981). Accordingly, our results emphasize that edelfosine affects the disease-relevant cell population with relative specificity. $CD8^+$ T cells in EAE may have regulatory functions (Friese and Fugger, 2005; Linker et al., 2005; Zeine and Owens, 1993). However, these cells also produce IFN-γ and

potentially serve as an early source of IFN-γ driving Th1-cell differentiation (Das et al., 2011). Treatment with edelfosine led to reduced frequencies of IFN-γ-producing $CD8^+$ T cells. In lymphocyte infiltrates of the CNS increased frequencies of $CD4^+$ T cells that expressed activated caspase-3 after edelfosine treatment were found. These data indicate that either edelfosine is able to induce apoptosis in T cells that have infiltrated the CNS of EAE mice or that the treatment is sufficient to induce apoptosis in T cells in the periphery, which may not prevent those cells from infiltrating the CNS but still leads to disease amelioration. As a summary result edelfosine treatment prevents neuronal loss and ameliorates EAE. Accordingly, immunohistochemical data correlated with disease scores.

Recently, we were able to show that edelfosine also inhibits homeostatic proliferation of human T cells, i.e. proliferation in the absence of an antigenic stimulus (Abramowski et al., 2014). While we did not examine this aspect in detail in the present study, the above observation of higher stimulation indices in antigen-specific proliferation studies due to reduced background proliferation (see Fig. 5B) indicates that inhibition of homeostatic proliferation also applies to murine cells. Further analyses in human T- and B cells demonstrated novel mechanisms of action of edelfosine including the downmodulation of MHC-class II molecules and the induction of type I IFN-associated genes (Abramowski et al., 2014). Here, we complemented these *in vitro* data with human cells and show the effectiveness of edelfosine treatment in EAE as well as the *in vivo* induction of apoptosis in disease-relevant T cell subsets. Our results underscore the potential of edelfosine to treat autoimmune diseases including MS.

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Figure Captions

Figure 1. EAE disease course in C57BL/6 and SJL mice was dependent on edelfosine treatment. (A) Whereas PBS-treated C57BL/6 mice developed the classical chronic-progressive EAE course, mice treated with edelfosine appeared to be impacted in their EAE development but also in their physical presentation due to side effects at concentrations higher than 10 mg/kg edelfosine. Clinical scores for groups of PBS-treated (n=3), 10 mg/kg edelfosine treated (n=3), 15 mg/kg edelfosine treated (n=2) and 25 mg/kg edelfosine treated (n=2) EAE mice. Note that the treatment of mice with 25 mg/kg and 15 mg/kg edelfosine led to drug-related side effects which possibly had an impact on EAE development in immunized mice that remained clinically inconspicuous. (B) The preventive administration of edelfosine every other day had no significant effect on RR-EAE clinical scores in immunized SJL mice. Depicted groups: PBS-treated (n=6), 1 mg/kg edelfosine treated (n=6) and 10 mg/kg edelfosine treated (n=6) EAE mice. (C) Clinical scores for groups of PBS-treated (n=3), 1 mg/kg edelfosine treated (n=4) and 10 mg/kg edelfosine treated (n=7) EAE mice with administration starting at disease onset (day 11). A treatment effect could not be proved. EAE experiments are shown as mean values ± SEM; *P<0.05, **P<0.01 after 2-way ANOVA. (D) No significant reduction in cumulative disease scores was determined for groups of immunized SJL mice that were treated every other day beginning at day 5 with 1 mg/kg edelfosine or 10 mg/kg edelfosine compared to PBS-treated mice. (E) The therapeutic treatment of EAE-induced SJL mice starting at disease onset (day 11) yielded cumulative disease scores that implied an edelfosine dose-dependent reduction compared to PBS-treated controls. However, significance could not be proved. The data that is based on EAE experiments whose corresponding disease courses are depicted in (B) and (C), respectively, are shown as mean values ± SEM; *P<0.05, **P<0.01 after post-hoc analysis.

Figure 2. Edelfosine application influenced EAE in SJL mice. (A) The preventive treatment of EAE-induced SJL mice with 10 mg/kg edelfosine on a daily basis ameliorated the disease course: depiction of clinical scores for groups of PBS-treated (n=7), 1 mg/kg edelfosine treated (n=7) and 10 mg/kg edelfosine treated (n=7) EAE mice. The EAE experiment is shown as mean values ± SEM; *P<0.05 after 2-way ANOVA. (B) Cumulative disease scores were reduced upon preventive edelfosine treatment of EAE-induced SJL mice. The daily administration of 10 mg/kg edelfosine to EAE-induced SJL mice , but not the administration of 1 mg/kg edelfosine , reduced the cumulative disease score compared to PBS-treated controls . The data that is based on EAE experiments whose corresponding disease courses are depicted in (A) are shown as mean values ± SEM; *P<0.05 after post-hoc analysis.

Figure 3. Increased frequency of naïve $CD4^+$ T cells after treatment with 10 mg/kg edelfosine. (A) Lymph node T cells of EAE-induced PBS- or edelfosine-treated SJL mice were analyzed for frequencies of $CD4^+$ and $CD8^+$ T cell subsets, but also for their expression of CD62L and CD44 on the cell surface. (B) $CD4^+$ and $CD8^+$ T cell frequencies remained unchanged irrespective of the treatment. (C) Increased frequencies of $CD62L^+CD44^-$ naïve $CD4^+$ T cells were detected in lymph nodes of mice that received 10 mg/kg edelfosine (1.22-fold). Frequencies from one representative of two independent EAE experiments (n=3 for each group in each experiment), fold-changes merged from two independent EAE experiments are shown as mean values ± SEM (□ PBS, ▭ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05 after post-hoc analysis.

Figure 4. Edelfosine treatment induced the upregulation of activated caspase-3. (A) Gating strategy for activated caspase-3 in $CD4^+$ and $CD8^+$ T cells as well as their activated, $CD69^+$

descendants. (B) Representative frequencies of $CD4^+$ and $CD8^+$ T cells as well as activated $CD4^+$ and $CD8^+$ T cells with activated caspase-3. Here, cells were prepared from lymph nodes. (C) Treatment of EAE-induced mice with 10 mg/kg edelfosine resulted in a significant increase in $CD4^+$ and $CD8^+$ T cell frequencies with activated caspase-3 which was also found for $CD8^+CD69^+$ T cells (spleens). Frequencies from one representative of two independent EAE experiments (n=3 for each group in each experiment), fold-changes merged from two independent EAE experiments are shown as mean values ± SEM (▭ PBS, ▭ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05 after post-hoc analysis.

Figure 5. Edelfosine treatment of EAE-induced mice did not compromise proliferation capacity of lymph node- or spleen-derived cells. (A) After daily treatment of EAE-induced mice with either PBS or edelfosine, lymph node cells and spleen cells (not shown) were prepared, restimulated *ex vivo* with Con A, anti-CD3 antibody or $PLP_{(139-151)}$ and cultured for 72 h (+ stimulus added, - controls, absence of stimulus). Each symbol represents the mean value of triplicate approaches. Results from one representative of two independent EAE experiments (n=3 for each group in each experiment). (B) The relative proliferative response of cells within each condition expressed as SI. Lymph node- as well as spleen-derived cells showed no significant differences comparing the SIs of cells that were prepared from mice that received PBS, 1 mg/kg edelfosine or 10 mg/kg edelfosine and challenged with the same stimulus. Graphs display merged results from two independent experiments (n=6).

Figure 6. Analysis of edelfosine treatment effects on T cell subsets. (A) The gating strategy allows to examine $CD4^+$ and $CD8^+$ T cell frequencies as well as $CD4^+CD25^+Foxp3^+$ nTregs. Treatment of mice with 10 mg/kg edelfosine resulted in a reduced frequency of $CD4^+$ T cells in the CNS in the acute phase of EAE compared to treatment with PBS or 1 mg/kg edelfosine. (B) No differences in the frequency of nTregs were seen in the CNS of mice treated with 10

mg/kg edelfosine in comparison to PBS-treated mice. Frequencies from one EAE experiment (n=4 for each group, except PBS-treated group: n=3), frequencies are shown as mean values ± SEM (□ PBS, ▒ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05, **P<0.01 after post-hoc analysis.

Figure 7. Edelfosine treatment affected cytokine production of T lymphocytes. (A) The production of IFN-γ and IL-17 by $CD4^+$ or $CD8^+$ T cells was analyzed after intracellular cytokine staining. Dead cells were excluded. (B) The treatment of mice with 10 mg/kg edelfosine led to a decrease in frequencies of IFN-γ-producing $CD8^+$ T cells compared to PBS-treated and 1 mg/kg edelfosine-treated mice. For IL-17-producing $CD4^+$ T cells the P-value was 0.0463, but no differences between groups were found in post-hoc analysis. Frequencies from one EAE experiment (n=4 for each group, except PBS-treated group: n=3), frequencies are shown as mean values ± SEM (□ PBS, ▒ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05, **P<0.01 after post-hoc analysis.

Figure 8. Increased caspase-3 activation upon application of edelfosine. (A) CD4, CD8 and CD69 allowed the investigation of caspase-3 activation induced by edelfosine treatment. (B) $CD4^+$ T cells with activated caspase-3 showed that treatment with 10 mg/kg edelfosine increased the frequency of this apoptosis-indicative population compared to respective cells from PBS-treated mice. Frequencies from one EAE experiment (n=4 for each group, except PBS-treated group: n=3), frequencies are shown as mean values ± SEM (□ PBS, ▒ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05 after post-hoc analysis.

Figure 9. Reduction of $NeuN^+$ neurons in the acute phase was prevented by edelfosine. (A) Immunized SJL mice were treated daily with PBS or edelfosine. CNS was prepared at day 14. The EAE course revealed a significant treatment effect with differences between PBS-treated

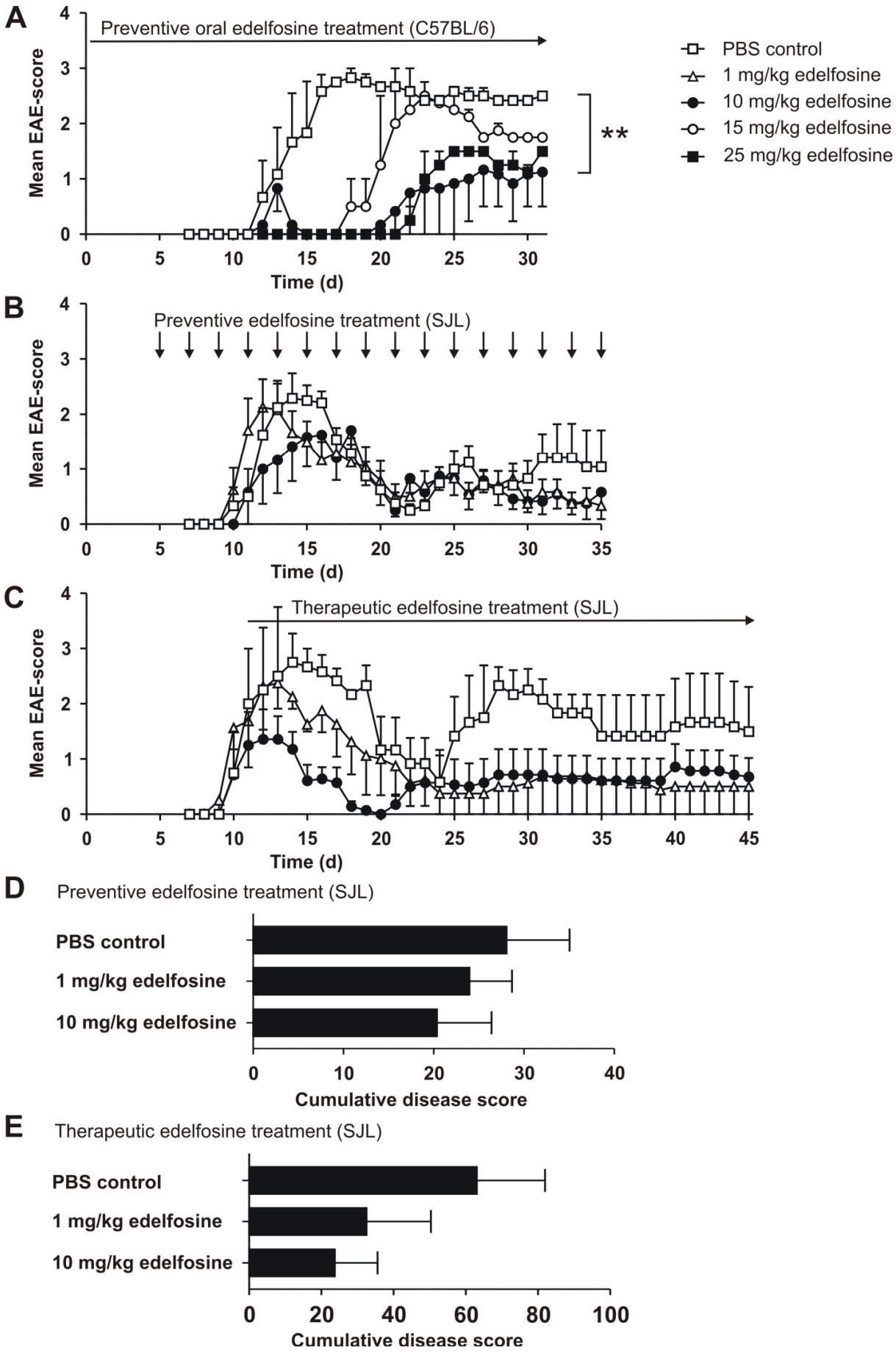
mice and mice that received either 1 mg/kg or 10 mg/kg edelfosine (day 11 to 13). (B) For each mouse, six sections of the cervical spinal cord were stained using an anti-NeuN antibody. Per section two photographs were evaluated for each of the two ventral horns. $NeuN^+$ neurons were counted to determine means. Compared to non-immunized, age-matched SJL mice PBS-treated EAE mice showed a reduced number of $NeuN^+$ neurons. This reduction could be prevented by treatment with either 1 mg/kg or 10 mg/kg edelfosine. (C) Representative photographs of cervical spinal cord sections stained for $NeuN^+$ neurons after treatment with either PBS, 1 mg/kg or 10 mg/kg edelfosine (scale bar represents 100 µm, magnification: 20x).

Supplementary Figure 1. Analysis of leukocyte subset frequencies. The specific expression of cell surface markers allowed discrimination between cell types as exemplified by this gating strategy depicting lymph node cells from an EAE-induced, 10 mg/kg edelfosine-treated mouse. Frequencies were determined by relating the detected number of events within the respective gate to the measured number of $CD45^+$ events.

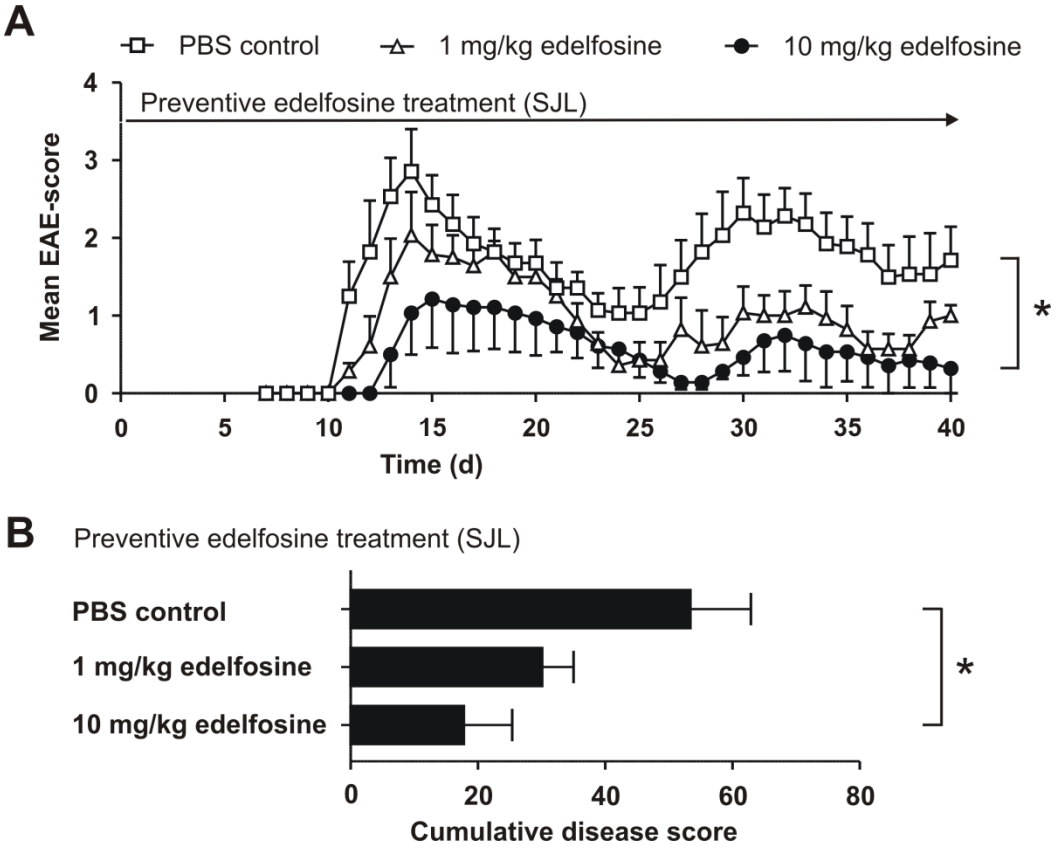
Supplementary Figure 2. Quantification of immune cell infiltration at the acute phase of EAE. (A) The acute phase of EAE was accompanied by a maximal clinical impairment of mouse movement. PBS-treated mice showed the expected development of EAE whereas the treatment of mice with 1 mg/kg edelfosine or 10 mg/kg edelfosine resulted in milder clinical EAE scores. In detail, a significant treatment effect was found with significant differences between PBS-treated and 1 mg/kg edelfosine-treated groups (day 14, 15) as well as between PBS-treated and 10 mg/kg edelfosine-treated groups (day 13 to 15). (B) Infiltrating $CD45^+$ immune cells into brains and spinal cords were prepared and quantified by flow cytometry. (C) Quantification of CNS-infiltrating $CD45^+$ leukocytes was performed by flow cytometry using BD Trucount tubes according to the displayed gating strategy. $CD45^+$ events were

related to detected bead events in the Fl-1/Fl-2-defined gate and the absolute number of infiltrating cells was calculated using the absolute bead number. (D) The treatment with 10 mg/kg edelfosine led to a significant reduction in T cell frequencies compared to PBS treatment. Frequencies from one EAE experiment (n=4 for each group, except PBS-treated group: n=3), frequencies are shown as mean values ± SEM (▭ PBS, ▭ 1 mg/kg edelfosine, ■ 10 mg/kg edelfosine); *P<0.05 after post-hoc analysis.

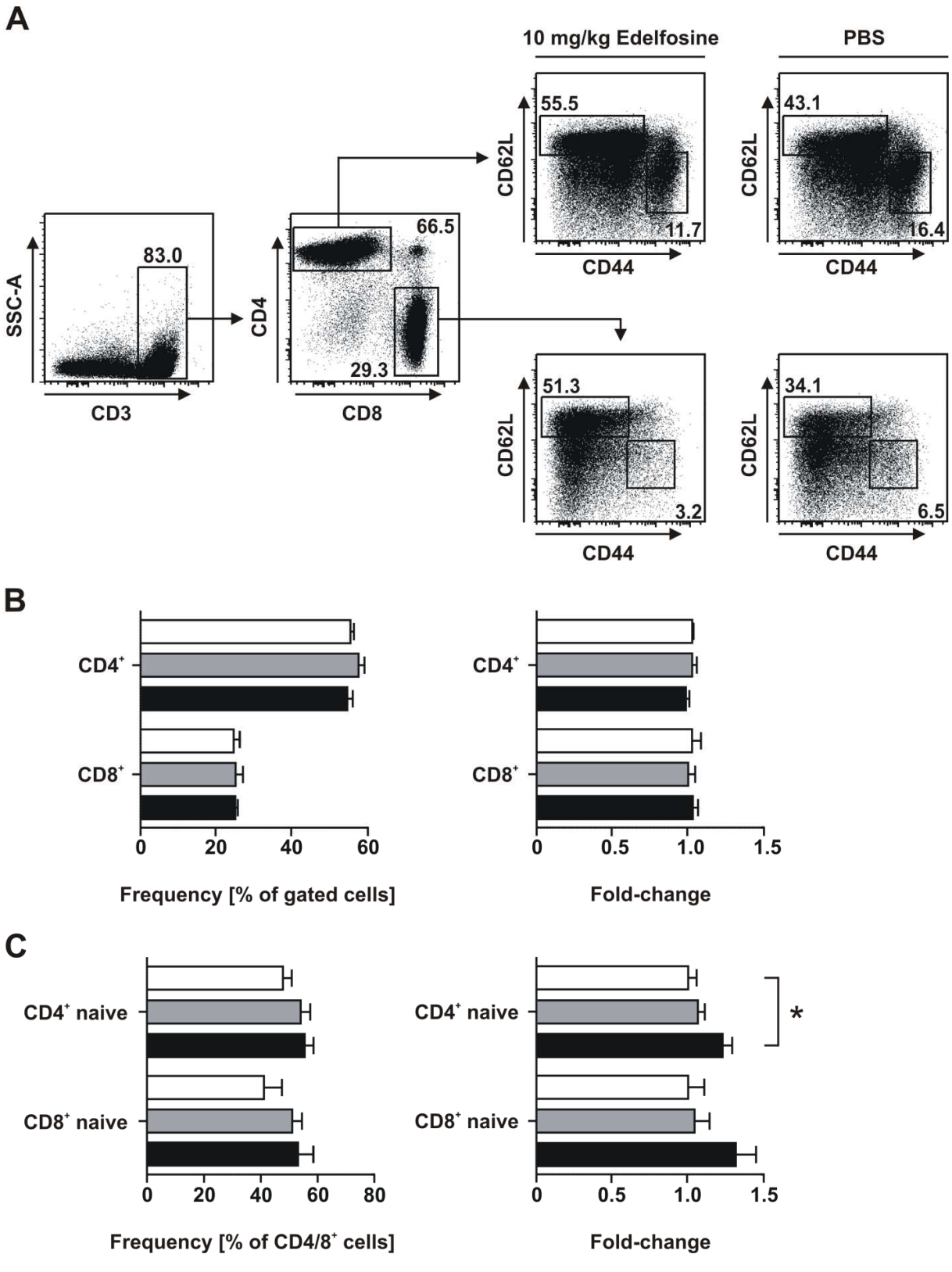
Supplementary Figure 3. Identification of infiltrating cells into the CNS of EAE-induced SJL mice in the acute phase due to the expression of characteristic surface marker molecules. After the separation of $CD45^{int}$ CNS-residing microglia from $CD45^{high}$ infiltrating leukocytes, the latter population was further specified (leukocyte subsets) as exemplified by this gating strategy depicting cells from an EAE-induced, 10 mg/kg edelfosine-treated mouse.

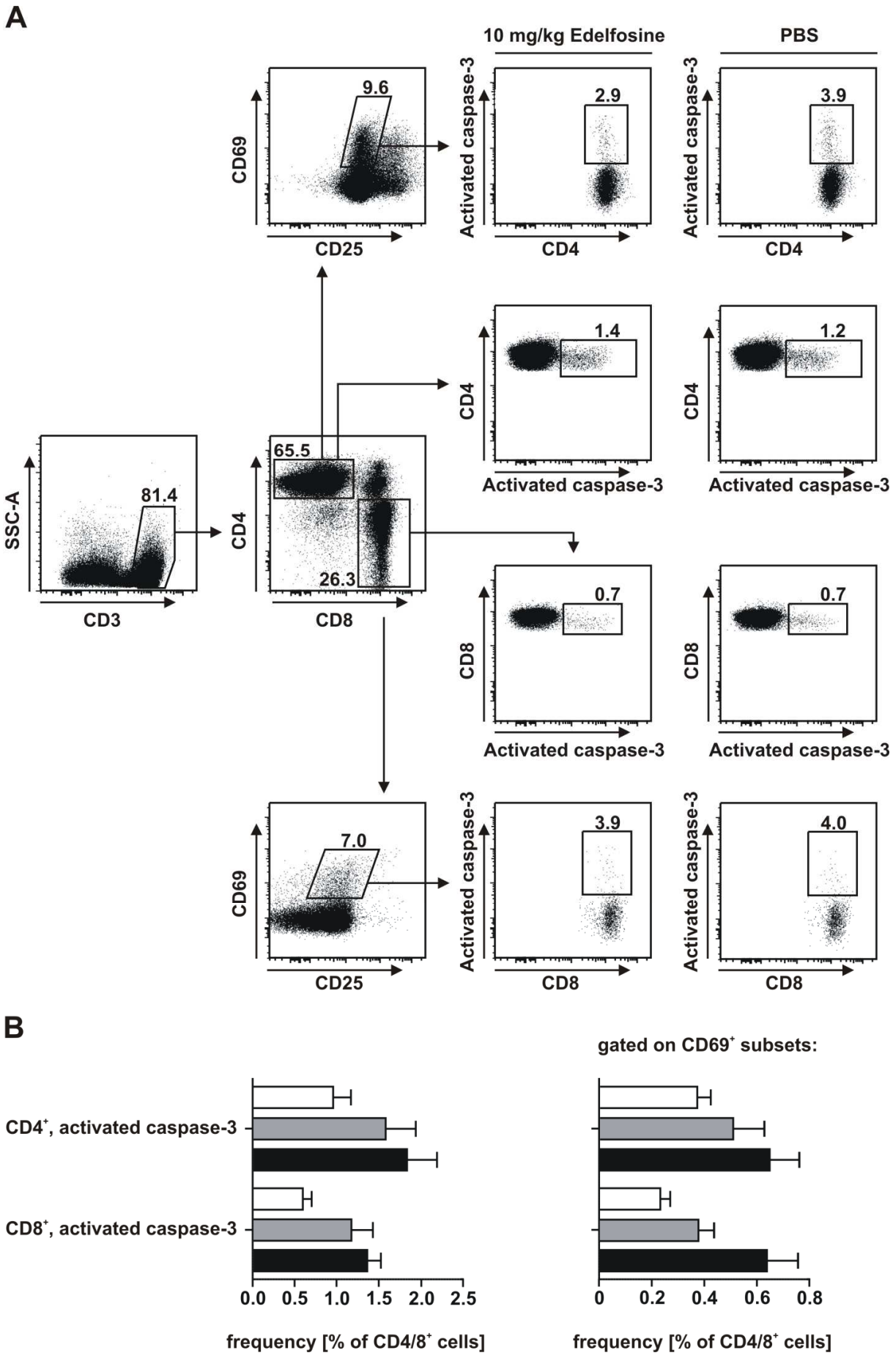

A
Preventive oral edelfosine treatment (C57BL/6)
Mean EAE-score
Time (d)
**
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
15 mg/kg edelfosine
25 mg/kg edelfosine
B
Preventive edelfosine treatment (SJL)
Mean EAE-score
Time (d)
C
Therapeutic edelfosine treatment (SJL)
Mean EAE-score
Time (d)
D
Preventive edelfosine treatment (SJL)
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Cumulative disease score
E
Therapeutic edelfosine treatment (SJL)
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Cumulative disease score

1


A
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Preventive edelfosine treatment (SJL)
Mean EAE-score
Time (d)
*
B
Preventive edelfosine treatment (SJL)
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Cumulative disease score
*

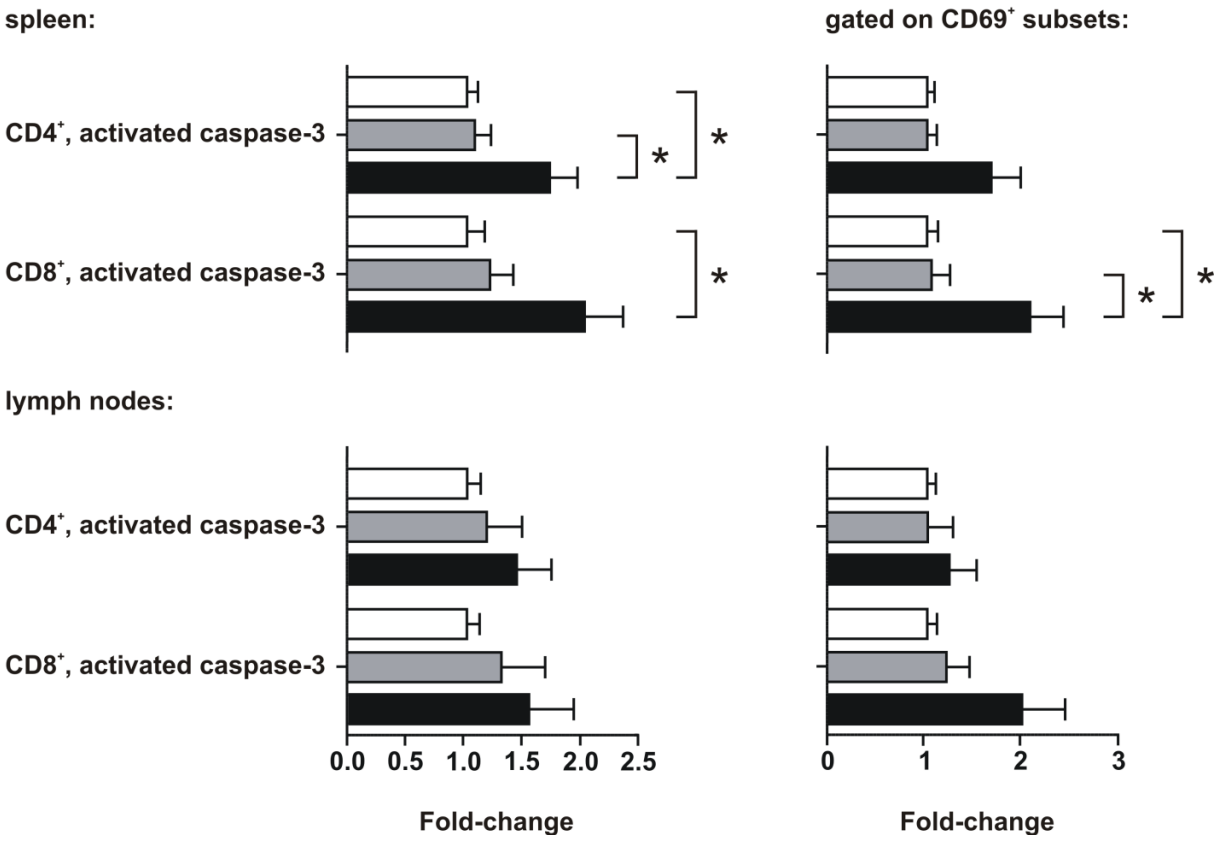
1


A
10 mg/kg Edelfosine
PBS
SSC-A
83.0
CD3
CD4
66.5
29.3
CD8
CD62L
55.5
11.7
CD44
43.1
16.4
51.3
3.2
34.1
6.5
B
CD4+
CD8+
0
20
40
60
Frequency [% of gated cells]
0.5
1.0
1.5
Fold-change
C
CD4+ naive
CD8+ naive
80
Frequency [% of CD4/8+ cells]
*

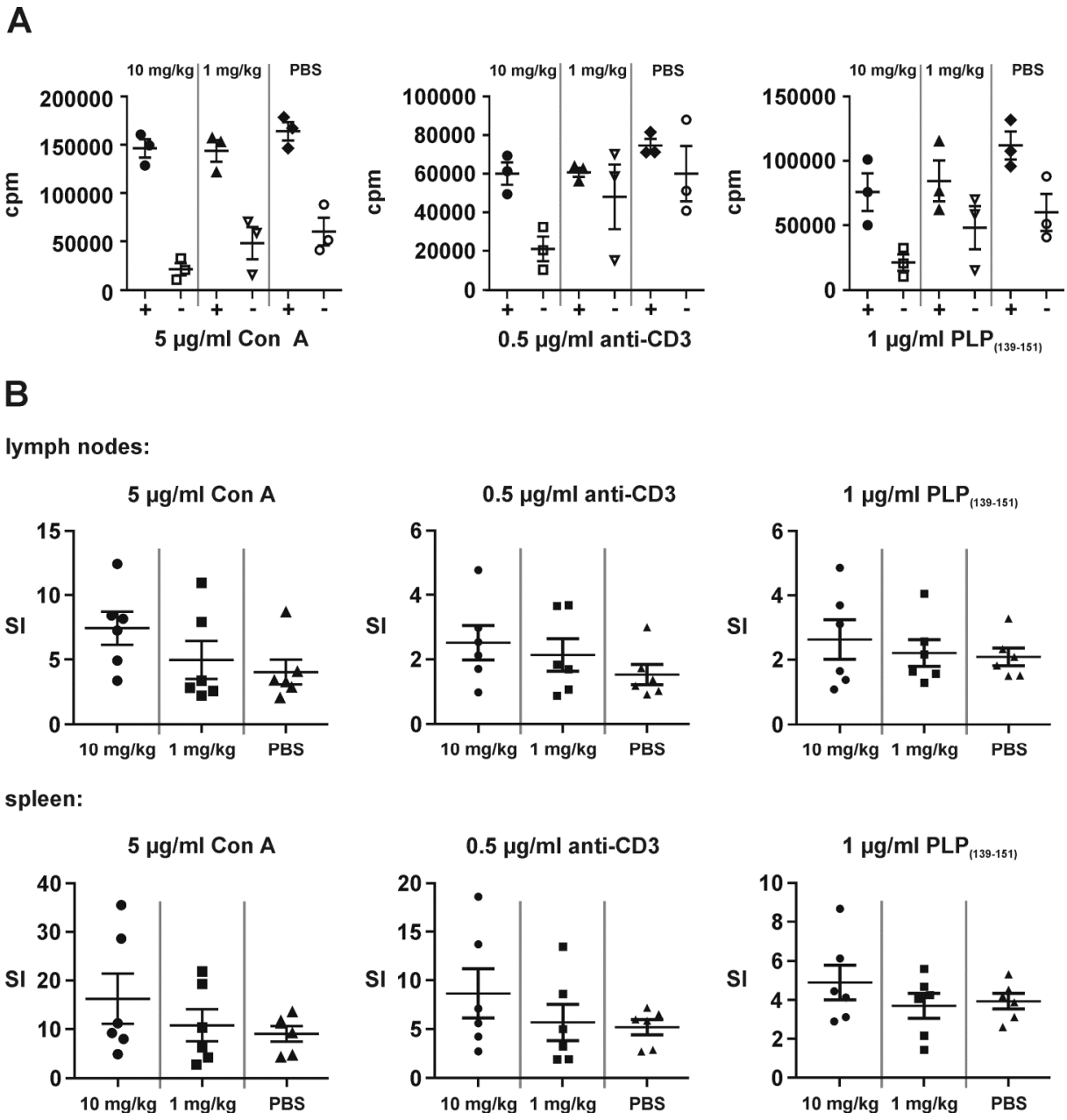

A
10 mg/kg Edelfosine
PBS
9.6
2.9
3.9
CD69
CD25
Activated caspase-3
CD4
1.4
1.2
81.4
65.5
26.3
SSC-A
CD3
CD8
0.7
7.0
3.9
4.0
B
gated on CD69+ subsets:
CD4+, activated caspase-3
CD8+, activated caspase-3
0.0 0.5 1.0 1.5 2.0 2.5
0 0.2 0.4 0.6 0.8
frequency [% of CD4/8+ cells]

1

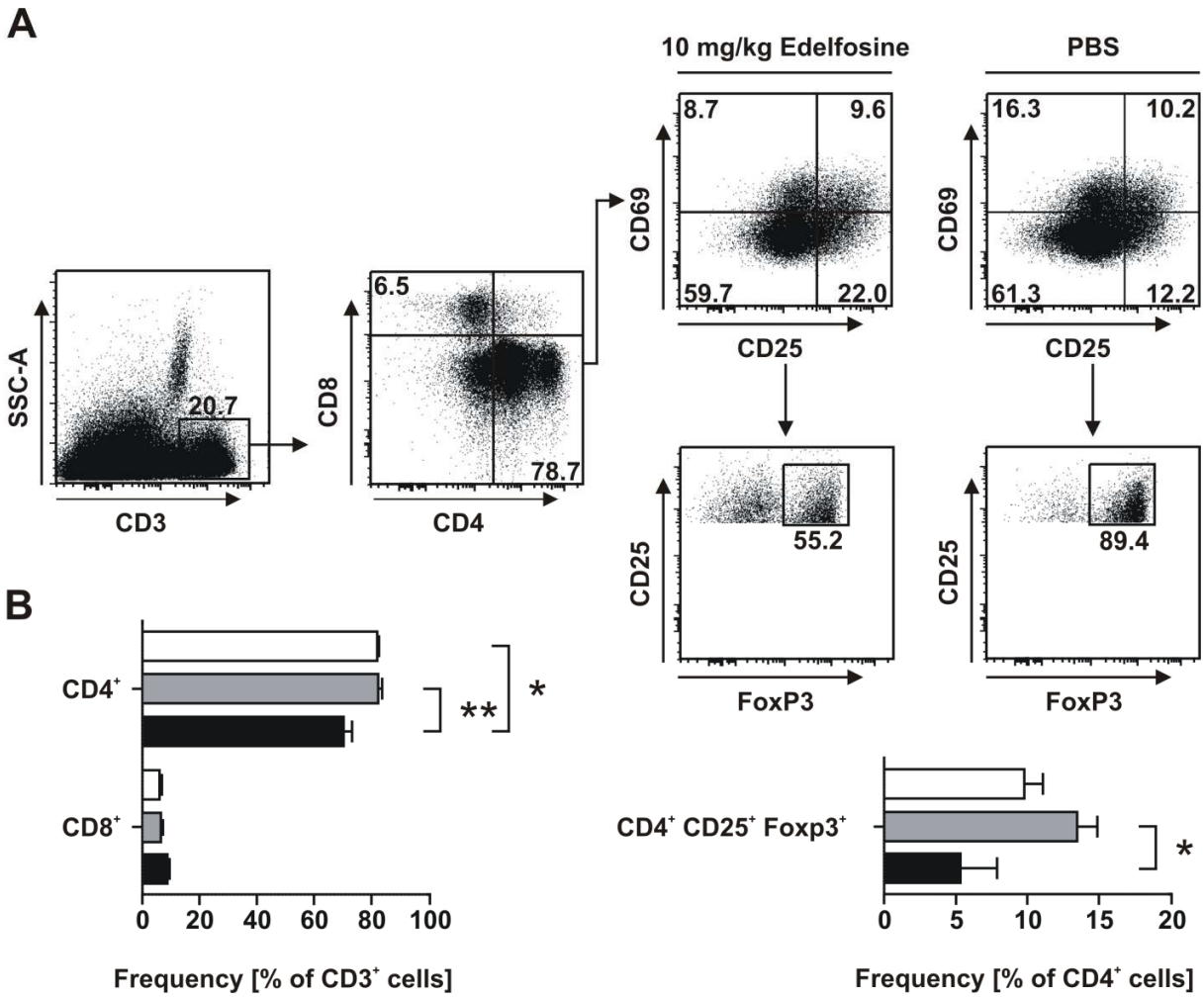
C


spleen:
gated on CD69+ subsets:
CD4+, activated caspase-3
CD8+, activated caspase-3
*
*
*
*
lymph nodes:
CD4+, activated caspase-3
CD8+, activated caspase-3
0.0 0.5 1.0 1.5 2.0 2.5
0 1 2 3
Fold-change
Fold-change

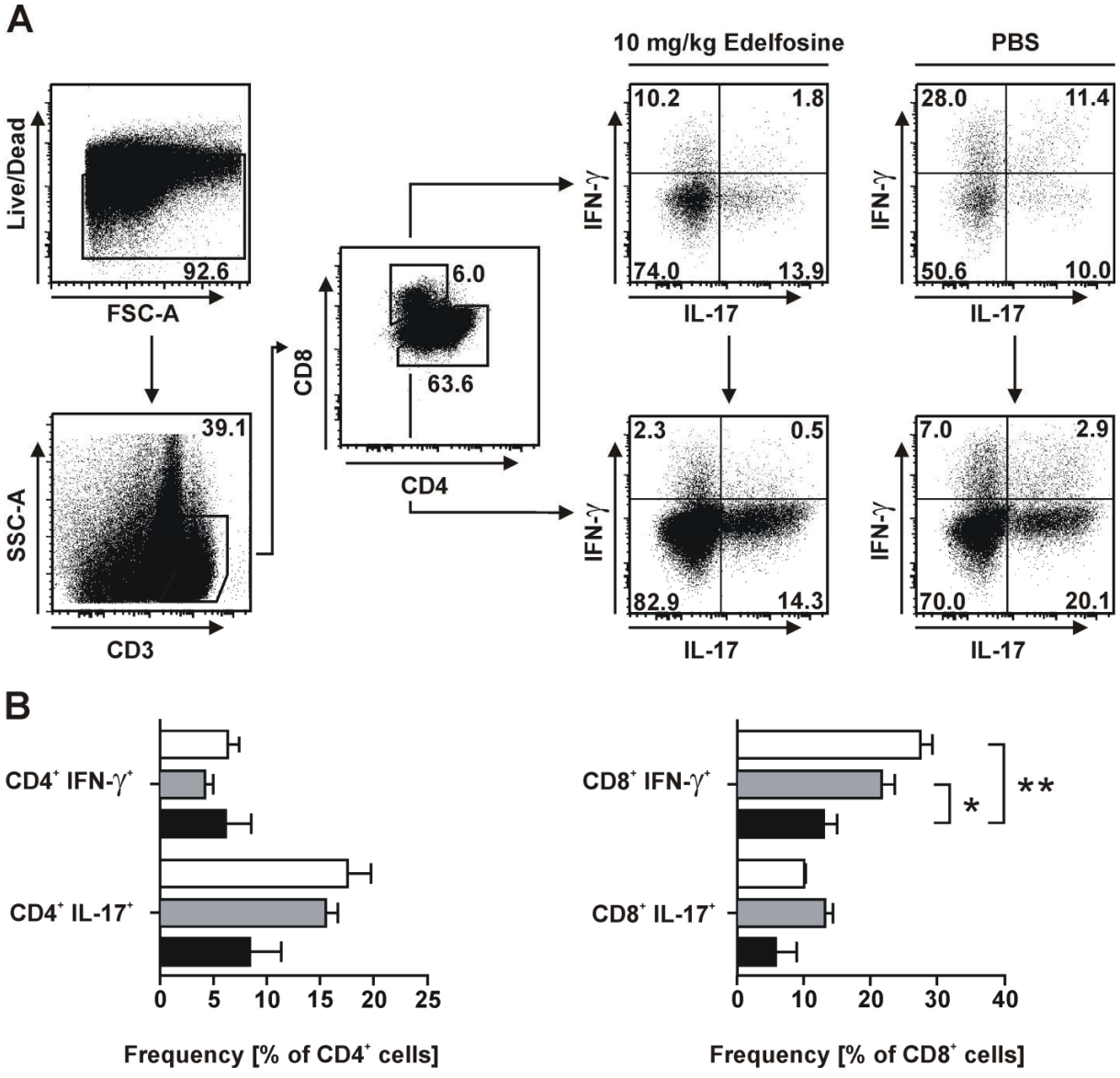
1


A
10 mg/kg
1 mg/kg
PBS
cpm
200000
150000
100000
50000
0
+ - + - + -
5 µg/ml Con A
100000
80000
60000
40000
20000
0.5 µg/ml anti-CD3
150000
100000
50000
1 µg/ml PLP(139-151)
B
lymph nodes:
5 µg/ml Con A
0.5 µg/ml anti-CD3
1 µg/ml PLP(139-151)
SI
15
10
5
6
4
2
10 mg/kg
1 mg/kg
PBS
spleen:
40
30
20
10
0
8

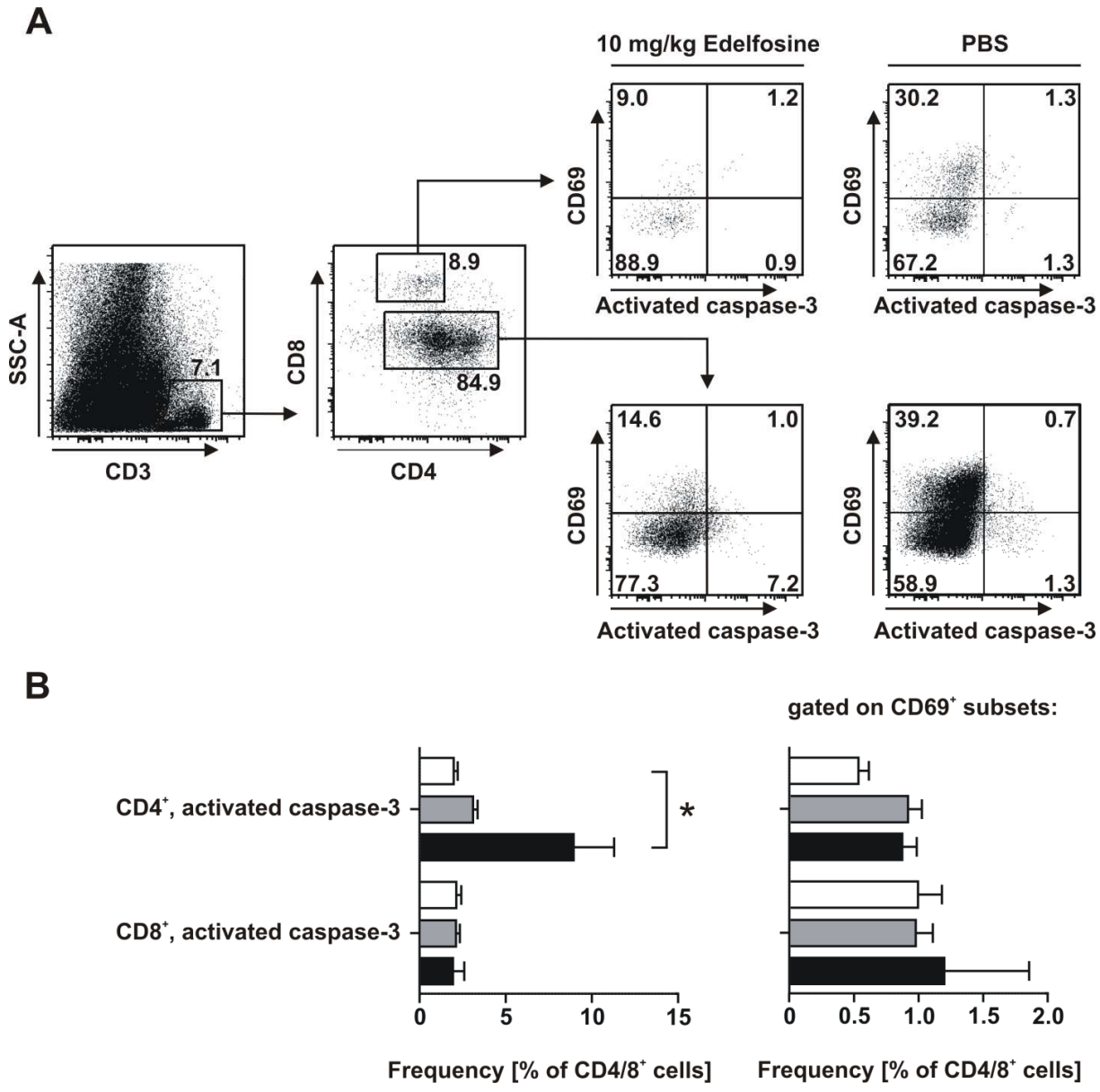
1


A
10 mg/kg Edelfosine
PBS
SSC-A
20.7
CD3
CD8
6.5
78.7
CD4
CD69
8.7
9.6
59.7
22.0
CD25
16.3
10.2
61.3
12.2
CD25
CD25
55.2
FoxP3
CD25
89.4
FoxP3
B
CD4+
CD8+
**
*
0
20
40
60
80
100
Frequency [% of CD3+ cells]
CD4+ CD25+ Foxp3+
*
0
5
10
15
20
Frequency [% of CD4+ cells]

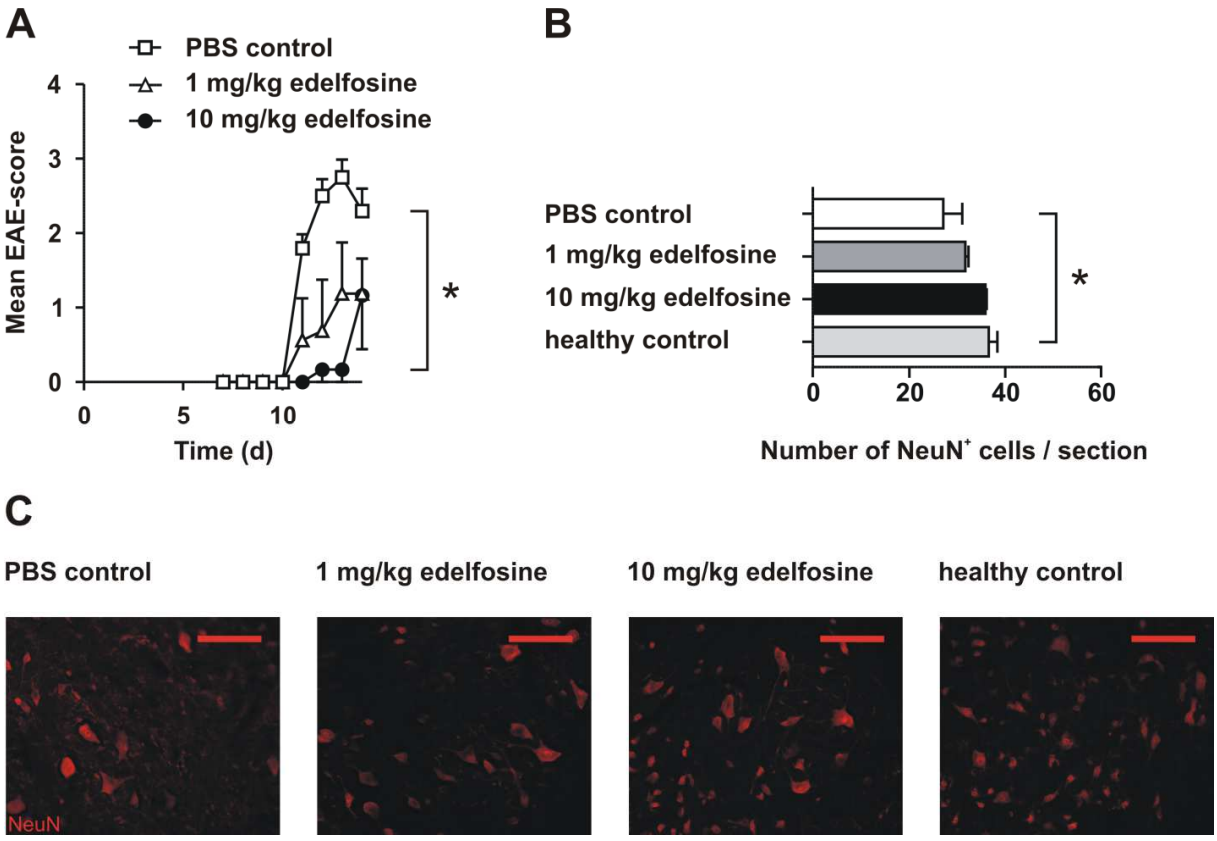
1


A
10 mg/kg Edelfosine
PBS
Live/Dead
92.6
FSC-A
SSC-A
39.1
CD3
CD8
6.0
63.6
CD4
IFN-γ
10.2
1.8
74.0
13.9
IL-17
28.0
11.4
50.6
10.0
2.3
0.5
82.9
14.3
7.0
2.9
70.0
20.1
B
CD4+ IFN-γ+
CD4+ IL-17+
0
5
10
15
20
25
Frequency [% of CD4+ cells]
CD8+ IFN-γ+
CD8+ IL-17+
*
**
0
10
20
30
40
Frequency [% of CD8+ cells]

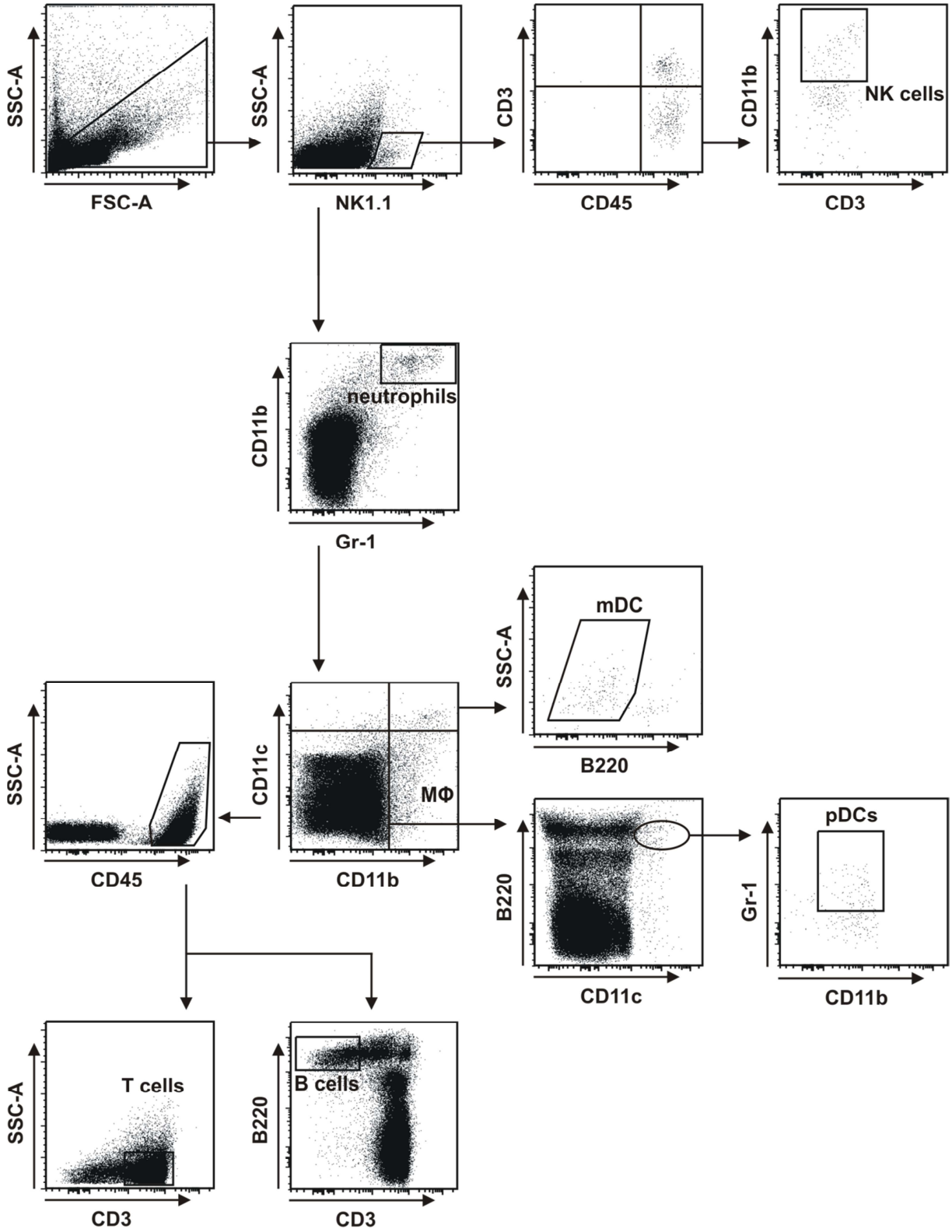
1


A
10 mg/kg Edelfosine
PBS
SSC-A
CD3
7.1
CD8
CD4
8.9
84.9
CD69
Activated caspase-3
9.0
1.2
88.9
0.9
30.2
1.3
67.2
1.3
14.6
1.0
77.3
7.2
39.2
0.7
58.9
1.3
B
gated on CD69+ subsets:
CD4+, activated caspase-3
CD8+, activated caspase-3
*
0
5
10
15
0
0.5
1.0
1.5
2.0
Frequency [% of CD4/8+ cells]
Frequency [% of CD4/8+ cells]

1


A
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Mean EAE-score
0
1
2
3
4
0
5
10
Time (d)
*
B
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
healthy control
0
20
40
60
*
Number of NeuN+ cells / section
C
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
healthy control
NeuN

1


SSC-A
FSC-A
SSC-A
NK1.1
CD3
CD45
CD11b
NK cells
CD3
CD11b
neutrophils
Gr-1
SSC-A
mDC
B220
SSC-A
CD45
CD11c
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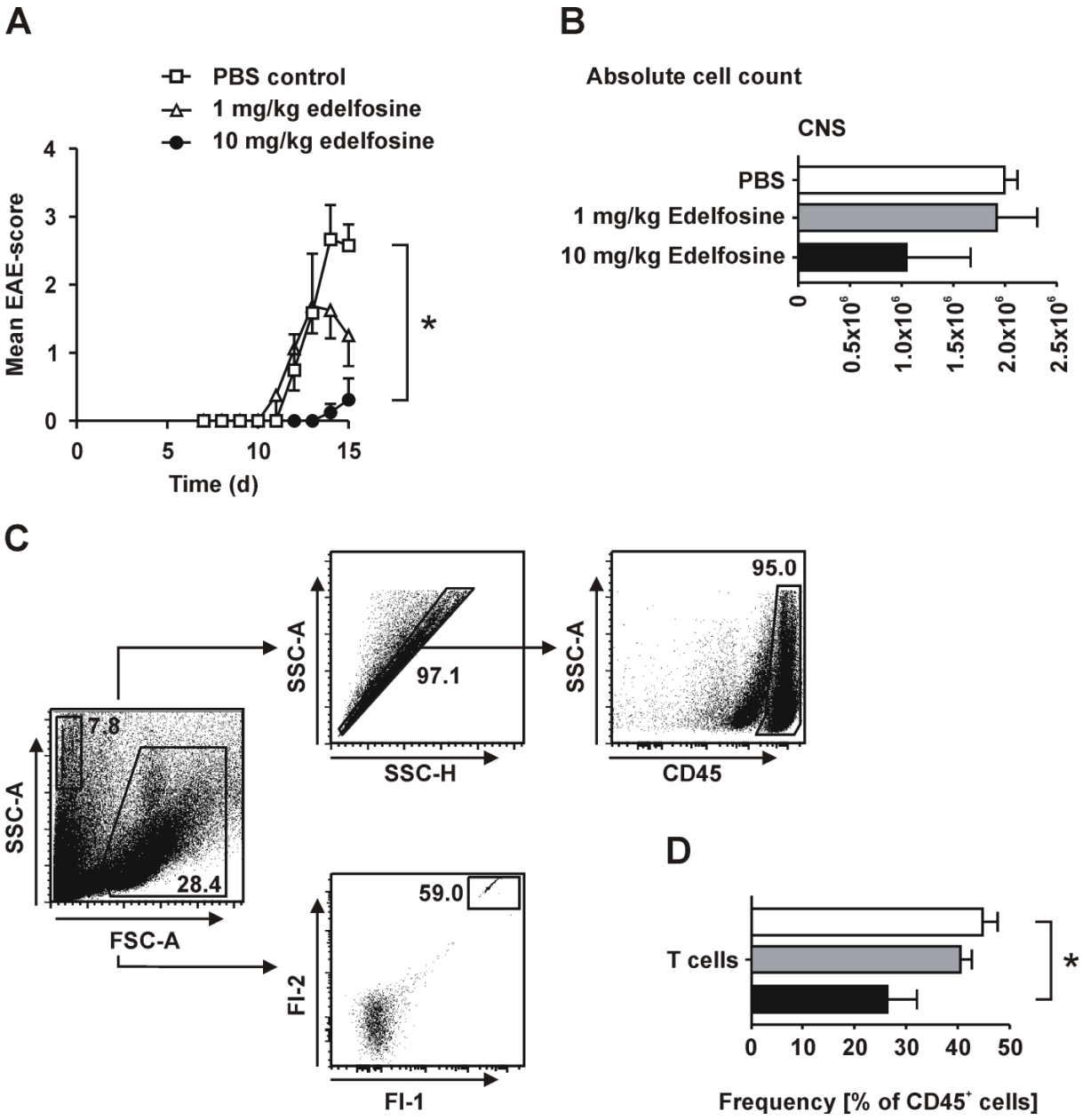
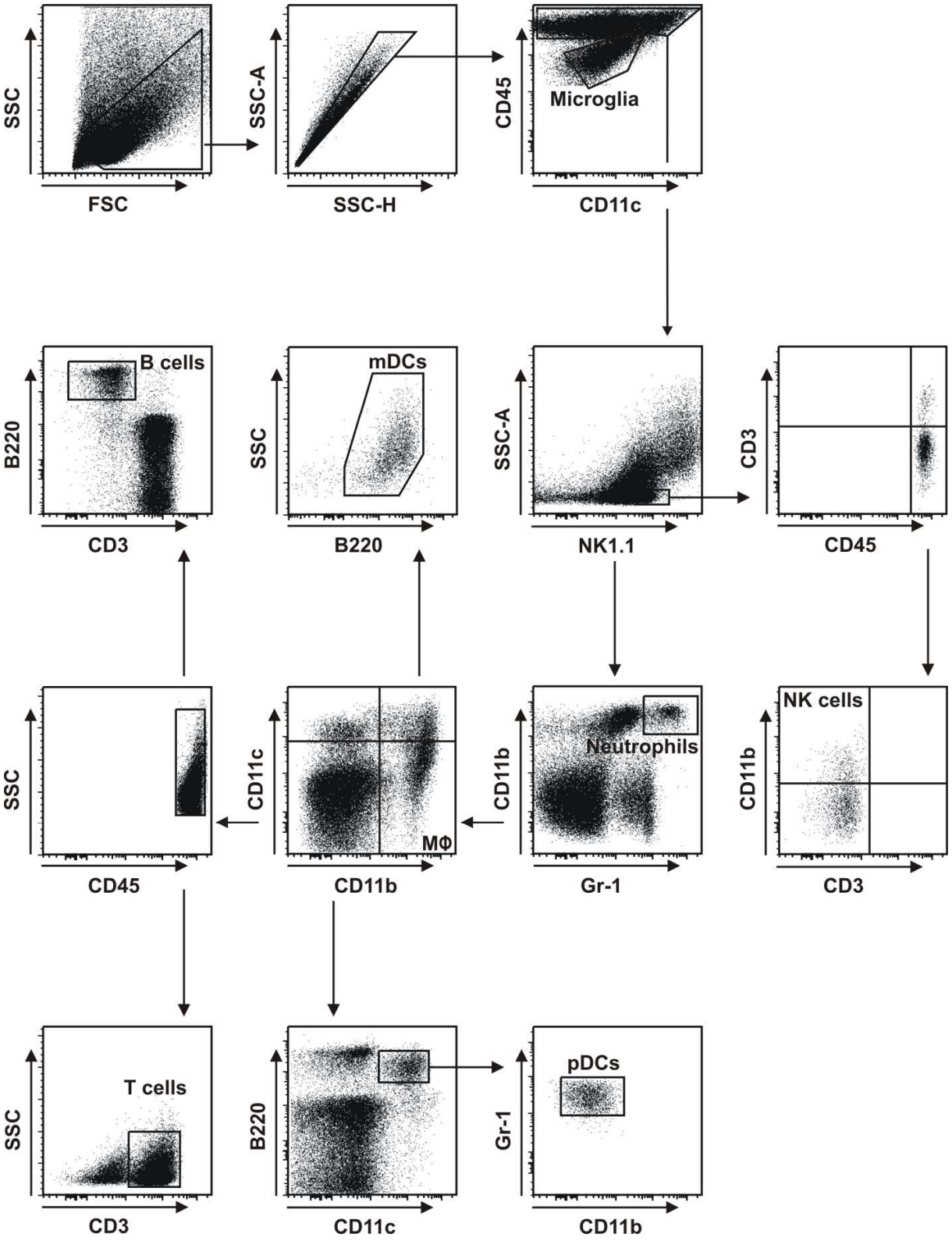
SSC-A
T cells
CD3
B220
B cells
CD3

1


A
PBS control
1 mg/kg edelfosine
10 mg/kg edelfosine
Mean EAE-score
Time (d)
*
B
Absolute cell count
CNS
PBS
1 mg/kg Edelfosine
10 mg/kg Edelfosine
C
SSC-A
FSC-A
7.8
28.4
SSC-H
97.1
CD45
95.0
FL-2
FL-1
59.0
D
T cells
Frequency [% of CD45+ cells]

1


SSC
FSC
SSC-A
SSC-H
CD45
Microglia
CD11c
B cells
B220
CD3
mDCs
SSC
B220
SSC-A
NK1.1
CD3
CD45
SSC
CD45
CD11c
MΦ
CD11b
CD11b
Neutrophils
Gr-1
NK cells
CD11b
CD3
SSC
T cells
CD3
B220
CD11c
Gr-1
pDCs
CD11b

1